Somatic Mitochondrial Mutation in Gastric Cancer

Lawrence J. Burgart,* Jian Zheng,†
Qiuping Shu,† John G. Strickler,* and
Darryl Shibata†

From the Department of Anatomic Pathology, Mayo Clinic,
Rochester, Minnesota, and the Department of Pathology,†
University of Southern California School of Medicine, Los
Angeles, California*

Likely hot spots for mutations are mitochondrial sequences as there is less repair and more damage by carcinogens compared with nuclear sequences. A somatic 50-bp mitochondrial D-loop deletion was detected in four gastric adenocarcinomas. The deletion included the CSB2 region and was flanked by 9-bp direct repeats. The deletion was more frequent in adenocarcinomas arising from the gastroesophageal junction (4/32, 12.5%) compared with more distal tumors (0/45). Topographical analysis revealed the absence of the deletion from normal tissues except in focal portions of smooth muscle in one case. In two cases, apparent mutant homoplasmy was present throughout two tumors, including their metastases. In the two other cases, the mutation was present in only minor focal portions (<5%) of their primary tumors. These findings document the presence of somatic mitochondrial alterations in gastric cancer, which may reflect the environmental and genetic influences operative during tumor progression. (Am J Pathol 1995, 147:1105–1111)

Gastric cancer likely occurs after the accumulation of multiple mutations in critical oncogenes and tumor suppressor genes. The exact number and types of mutations necessary for malignant transformation are unknown. Aside from loci such as c-K-*ras*,[1,2] p53,[3–5] E-cadherin,[6] and multiple microsatellites in tumors with a mutator phenotype,[7–9] the mutations in gastric cancer are largely uncharacterized.

Mutations in cancer may arise because they provide selective value or because some loci are more prone to alteration. One likely hot spot for mutations is mitochondrial DNA (mtDNA) as it is preferentially modified by many carcinogens[10–13] and its repair[11,14,15] is less efficient compared with nuclear DNA. Because there can be several thousand mitochondrial genomes per cell,[15] it is unlikely that most mitochondrial mutations are immediately deleterious or specifically contribute to tumorigenesis. However, the types of mitochondrial mutations may reflect underlying genetic and environmental influences on the malignant clone, similar to the relative specificity of p53 mutations exhibited by different cancer types.[16] In this paper, we demonstrate in some gastric cancers a mitochondrial D-loop alteration.

Materials and Methods

Tumor Tissues

The 77 primary gastric adenocarcinomas were from gastrectomies performed at the Mayo Clinic. DNA was extracted from paraffin-embedded tissues as previously described.[9] Gastroesophageal tumors are defined as adenocarcinomas involving the gastric cardia.

Analysis of Mitochondrial D-Loop

A 445-bp portion of the human mitochondrial D-loop (bases 75 to 520 as numbered by Anderson et al[17]) was amplified with four sets of overlapping polymerase chain reaction (PCR) primers from the composite tumor sections. The primers that detected the deletion are SB317 (5' TCCACACAGACATCATA-ACA, upstream) and SB318 (AAAGTGCATACCGC-CAAAAG, downstream) and normally yield a 155-bp PCR product (mitochondrial bases 270 to 425[17]). PCR was performed for 44 cycles (95°C for 60 sec-

A


```
        mtTF-X                    CSB2                                                              CSB3
ACATCATAACAAAAAATTTCCA CCAAACCCC CCCCTCCCCCCGCTTCTGGCCACAGCACTTAAACACATCTCTG CCAAACCCC AAAAACA
```

DELETION

B

```
                                                    GAACCAACCAAACCCCAAAGACA ‾‾‾ HSP
                                                    | |||||||||||| |||
                          mtTF-X                                      CSB3
BREAKPOINT SEQUENCE --- ACATCATAACAAAAAATTTCCA CCAAACCCCAAAAACA ---
                        |  |||| | |  | |↑| | |||
                        CGCTG CTAAC CCCATACCCCGAACCAAC  mtTF-H
```

C

Figure 1. **A:** *The mitochondrial D-loop 50-bp deletion detected in the gastric cancers involves 9-bp direct repeats and deletes CSB2.* **B:** *The breakpoint sequence places a mitochondrial transcription factor binding site (mtTF-X) into CSB3. Sequence homologies between HSP and CSB3 and between mtTF-X and mtTF-H are noted.* **C:** *The deletion potentially activates CSB3 to a transcription start site, similar to HSP.*

onds, 58°C for 50 seconds, and 72°C for 60 seconds) in the presence of [³³P]dCTP (DuPont, Wilmington, DE). The labeled PCR products were analyzed by SSCP as previously described.[9] The aberrant bands were cut out from the acrylamide gel, cloned, and sequenced.[9]

Topographical Analysis for the Mitochondrial Deletion

Selective ultraviolet radiation fractionation (SURF) was performed as previously described[9,18] for the four tumors with the deletion. From each tumor or normal fraction (approximately equal to 50 cells), PCR was performed for 44 cycles as above either with or without labeled dCTP. The presence of the deletion in each region was confirmed either with SSCP gel analysis, size analysis with a sequencing gel, or dot blot analysis and ³²P-labeled allelic specific hybridization probes. The sequence for the deletion probe was TTTCCACCAAACCCCAAAAA. The topographical distributions were analyzed at least twice from independent tissue sections to verify the locations of the deletions.

Analysis for Non-D-Loop Deletion Associated with Aging

A 4977-bp mitochondrial deletion involving coding regions will yield a PCR product only when the deletion is present.[19] PCR was performed with primers MT1 and MT2[19] for 52 cycles and the PCR products were analyzed with a 1% ethidium-bromide-stained agarose gel. The positive control was DNA extracted from the heart of an elderly individual, known to have low levels of this deletion.[19]

Results

A portion of the human mitochondrial D-loop was amplified from 77 gastric adenocarcinomas and then examined with SSCP. In four cancers, similar but altered bands distinctly different from the patterns obtained from adjacent normal tissue were observed. These altered bands were cloned and sequenced to reveal identical 50-bp deletions that involved flanking 9-bp direct repeats (Figure 1). This deletion eliminates a functional region (conserved sequence block 2 or CSB2[20]) but places a mitochon-
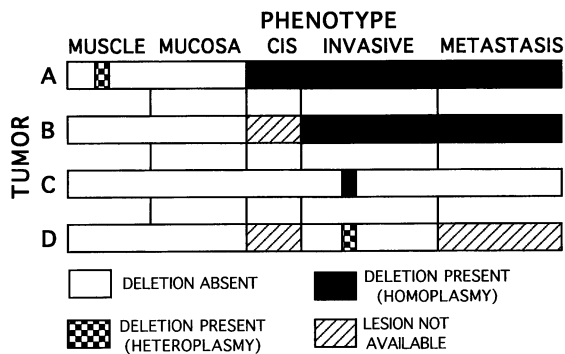
Figure 2. *Topographical distribution of the mitochondrial deletion in the four gastric adenocarcinomas. The metastasis in tumor A represents a recurrence in the adrenal, resected 1 year after the gastrectomy.*

drial transcription factor binding site (mtTF "X"[21]) into CSB3. The presence of this deletion in the tumors was confirmed by independent PCR assays with allelic specific hybridization probes, size analysis on sequencing gels, and SURF (see below). This deletion has not been reported as a normal polymorphism.[22] A larger 4977-bp deletion of coding mtDNA associated with flanking direct repeats and aging[19] could not be detected in the tumors with the D-loop deletion.

To further characterize this deletion, its topographical distribution was determined by SURF (Figure 2). The microdissection of tumors into small 200- to 400-cell fractions by SURF facilitates analysis of relatively pure (>70%) tumor cells from different tumor regions. In tumors A and B, the deletion was homogeneously present throughout the primary tumor and all metastases (Figure 3). The deletion appeared to be homoplasmic in the tumor cells as it was their predominant mitochondrial species. Small amounts of wild-type mitochondrial sequences could be detected in tumor fractions and are presumed to originate from contaminating normal cells. However, the possibility of minor tumor heteroplasmy cannot be strictly eliminated. The deletion was not detected in normal lymphocytes or mucosa, but a small amount was present in less than 10% of the smooth muscle fractions of tumor A (Figure 3).

In tumors C and D, the deletion was absent from most of the primary tumor and all metastases. The deletion could be detected only from focal, small portions (approximately 5%) of these primary tumors (Figure 4). In tumor C, the deletion appeared to be the predominant mitochondrial species in some of the malignant cells (Figure 4). The focal portions with the deletion were present in the submucosa of the primary tumors and did not have histological features distinctive from the bulk of tumor without the
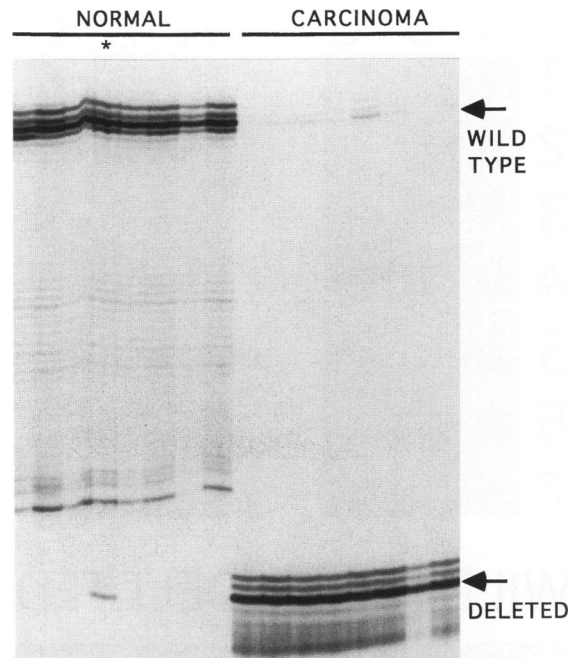

Figure 3. *Size analysis of labeled mitochondrial PCR products from different carcinoma or normal (mucosa and stroma) regions of tumor A (6% acrylamide sequencing gel). The deletion is evident in every carcinoma region and absent from the normal tissue regions except for a small amount present in one smooth muscle fraction (*). The deletion is the predominant mitochondrial species in the carcinoma, and the small amount of the wild-type sequences may reflect contamination by normal cells in the tumor fractions or residual wild-type sequences in the tumor cells.*

deletion (Figure 5). The deletion could not be detected from the normal tissues of these patients.

All four gastric adenocarcinomas with the deletion arose at the gastroesophageal junction. Its incidence was significantly higher ($P = 0.0257$) in tumors arising at the gastroesophageal junction (4 of 32, 12.5%) compared with more distal gastric adenocarcinomas (0 of 45). The clinical and pathological features (Figure 5) of the four tumors were otherwise not distinctive (Table 1). The tumors did not have prominent eosinophilic granular (oncocytic) cytoplasm. Interestingly, the patient with tumor B is still alive 13 years after gastrectomy with stage III disease. Microsatellite instability, the presence of Epstein-Barr virus, and p53 or c-K-*ras* mutations were not detected in these tumors (Table 1).

Discussion

Mitochondrial mutations may reflect the environmental and genetic backgrounds of a tumor. The mitochondrial genome is approximately 16,000 bases in size with several thousand genomes per cell. Up to 80% of these genomes can be mutated without phenotypic consequences,[23] and it has long been observed that some tumors predominantly utilize gly-
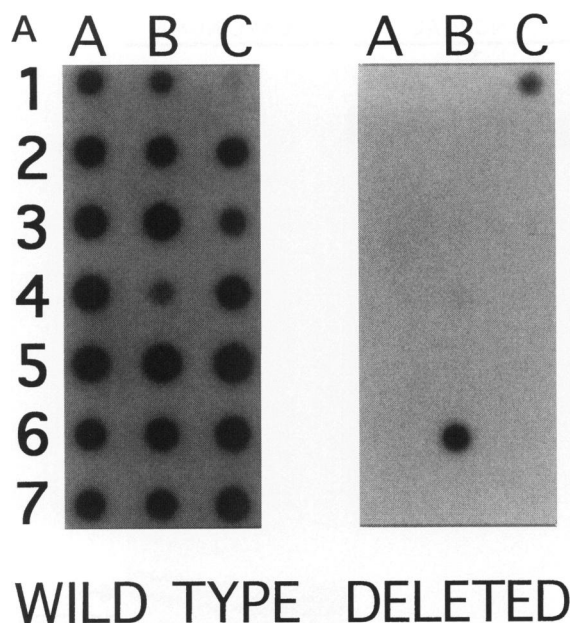
WILD TYPE   DELETED

Figure 5. *Histology of the tumors with mitochondrial deletions.* **A**: *Tumor A (H&E; magnification, ×400).* **B**: *Focal region of tumor C with the deletion (H&E; magnification, ×200). Oncocytic features are absent. Note that normal stromal cells are intimately present within the tumors and may represent the source of the normal mitochondrial sequences present in small amounts in the tumor fractions.*

Figure 4. **A**: *Dot blot analysis of different fractions from a single tissue slide of tumor C. Some of the different fractions are illustrated in a digital image (**B**) of the tumor and its protective dots (magnification, ×20). The labels correspond between the dot blot and the digital image with only regions B6 and C1 having the mitochondrial deletion. Evidence of homoplasmy for the deletion is evident in C1 as virtually no wild-type sequences are evident.*

colysis[24] or even totally lack mitochondria.[25] Therefore, deleterious mitochondrial mutations can potentially accumulate in cancers without immediate negative selection.

Mitochondrial sequences also evolve 10 to 17 times faster than comparable nuclear sequences.[23] Factors contributing to its more rapid evolution include a limited capacity to repair mtDNA[14,15] and its replication by the more error-prone DNA polymerase-γ.[15,26] In addition, mtDNA appears to be more vulnerable to damage by carcinogens.[27] Cell culture studies have demonstrated more DNA damage (adduct formation) that is repaired less completely than nuclear DNA.[10-13] The high lipid content (thought to concentrate hydrophobic carcinogens) and high concentration of reactive oxygen species in
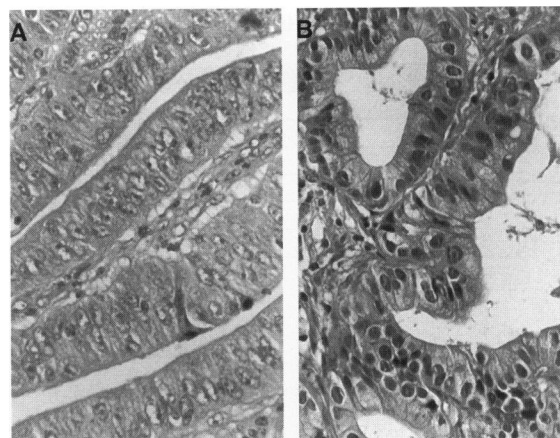
mitochondria may account for the increased damage.[12,13,28]

Despite these features, mitochondrial mutations have not been commonly detected in cancers. In general, homoplasmy (the same mtDNA in all cells) has been found in humans.[29] Heteroplasmy has been detected in rare myopathies associated with mitochondrial-related diseases.[23] In normal individuals, one general exception are large deletions that increase in frequency with aging, primarily in nonmitotic (brain and heart) tissues.[19,30] However, the frequency of this deletion is low (1/1000 mtDNA copies or less). Mitochondrial mutations in human solid tumors have been reported in isolated case reports.[31,32] However, one case involved large 7000-bp deletions present in surrounding cirrhotic liver but absent from the hepatic cancer.[31] The other example of two renal oncocytomas was characterized only as an extra band with Southern blot analysis[32] and has not been further characterized or confirmed.[33] One recent study sequenced a portion of the mitochondrial D-loop in colorectal cancers and failed to find mutations.[34]

The etiologies of the D-loop deletions and conversion to mutant homoplasmy are unknown. Slippage during DNA replication is a likely mechanism as direct repeats were present at the deletion sites.[35] Large human mtDNA deletions also commonly involve flanking direct repeats.[36] Small deletions involving direct repeats have been observed in mitochondrial mutator strains of petite yeast,[37] suggesting a possible genetic basis for the observed human deletions. Interestingly, the yeast nuclear genes, MSH1 and MSH2, are in-

Table 1. *Clinical Information*

Tumor	Size (cm)	Grade	TNM	Stage	Clinical data	Other mutations			
						USM	p53	c-K-*ras*	EBV
A	10×4×2	4	T2N0M0	2	75 WM, alive with disease (26 months)	—	—	—	—
B	7×6×1	3	T4N2M0	3	66 WM, alive without disease (13 years)	—	NT	NT	—
C	8×5×2	3	T2N3M1	4	48 WM, dead with disease (21 months)	—	—	—	—
D	6×4×1	4	T2N1M0	3	66 WM, dead with disease (46 months)	—	NT	NT	—

Clinical data include age (in years), race and gender, and status. Other mutations were determined as previously described. USM, ubiquitous somatic mutations[9]; p53[9]; c-K-*ras*[44]; EBV, Epstein-Barr virus[45]. WM, white male; NT, not tested.

volved, respectively, in mitochondrial and nuclear DNA repair.[38] The D-loop deletions were more frequent in proximal gastric adenocarcinomas, which have a distinct epidemiology compared with more distal cancers.[39] The mechanism for the conversion from presumed heteroplasmy to mutant homoplasmy is also unknown but may involve a replicative advantage for the deletion or could occur if only a fraction of all mtDNA copies in a cell are selected for replication. Segregation of heteroplasmic yeast to homoplasmy occurs after several mitotic divisions.[38]

The possibilities that this deletion represents conversion from minor germline heteroplasmy or normal changes that occur in the stomach or with aging cannot be strictly eliminated. However, except for one tumor, the deletion was not detectable in normal tissues. In this tumor, the very low levels of the deletion in smooth muscle but not normal mucosa may reflect a genetic propensity for the mutation or systemic exposure to a carcinogen. The D-loop deletions were also not associated with a general mitochondrial deletion aging phenomenon.[19,30]

The D-loop was targeted for mutational analysis becaue it is the only noncoding portion of the mitochondrial genome. Therefore, the D-loop has less constraints on the types of alterations it may acquire. However, the D-loop does contains essential transcription and replication elements (Figure 1). Interestingly, the 50-bp deletion largely preserved these elements. *In vitro* analysis has identified three conserved sequence blocks (CSBs) associated with heavy strand replication[40] and a heavy strand promoter (HSP) and light strand promoter for the transcription of the heavy and light mitochondrial strands.[41] Replication initiates with a RNA primer at the light strand promoter with subsequent DNA synthesis from CSB1, -2, or -3.[40,42] As the deletion eliminated CSB2, the persistence of the mtDNA lacking CSB2 implies that CSB1 and CSB3 alone can sup-

port replication. The deletion may have also created a new functional HSP from CSB3 (Figure 1). CSB3 has strong homology to HSP although significant transcription from CSB3 has not been observed.[41] The juxtaposition of a mtTF binding site into CSB3 may unmask its potential and allow transcription. The exact functional consequences of this deletion is unclear and currently under investigation.

The topographical distributions of the deletion provide insight into the evolution of their tumors. In tumors C and D, the deletion identified minor clonal tumor subpopulations that did not invade through the muscularis or metastasize. The deletion likely arose during clonal expansion and suggests that its selective value to these tumors is low. In contrast, for tumors A and B, the deletion appeared to be acquired before the bulk of clonal expansion and persisted in the invasive and metastatic portions. The potential of the transformed clones may be limited by acquiring apparent homoplasmy of the deletion very early in tumor progression.

The study of human tumors is often limited to single observations. The topographical distribution of tumor-associated mitochondrial mutations may improve the ability to dissect and identify the multiple populations that must occur during multi-step tumor progression.[43] Other types of somatic D-loop alterations have also been observed in gastric and lung cancers (unpublished data), and the specific mitochondrial changes may indicate the types of environmental and genetic influences operative during tumor progression.

Acknowledgments

References

1. Kihana T, Tsuda H, Hirota T, Shimosato Y, Sakamoto H, Terada M, Hirohashi S: Point mutation of c-Ki-*ras* oncogene in gastric adenoma and adenocarcinoma with tubular differentiation. Jpn J Cancer Res 1991, 82:308–314

2. Jiang W, Wahn SM, Guillem JG, Lu SH, Weinstein IB: Rapid detection of *ras* oncogenes in human tumors: applications to colon, esophageal, and gastric cancer. Oncogene 1989, 4:923–928

3. Yamada Y, Yoshida T, Hayashi K, Sekiya T, Yokota J, Hirohashi S, Nakatani K, Nakano H, Sugimura T, Terada M: p53 gene mutations in gastric cancer metastases and in gastric cancer cell lines derived from metastases. Cancer Res 1991, 51:5800–5805

4. Kim JH, Takahashi T, Chiba I, Park JG, Birrer MJ, Roh JK, Lee HD, Kim JP, Minna JD, Gazdar AF: Occurrence of p53 gene abnormalities in gastric carcinoma tumors and cell lines. J Natl Cancer Inst 1991, 83:938–943

5. Tamura G, Kihana T, Nomura K, Terada M, Sugimura T, Hirohashi S: Detection of frequent p53 mutations in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. Cancer Res 1991, 51:3056–3058

6. Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR, Hofler H: E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res 1994, 54:3845–3852

7. Han HJ, Yanagisawa A, Kato Y, Park JG, Nakamura Y: Genetic instability in pancreatic and poorly differentiated type of gastric cancer. Cancer Res 1993, 53:5087–5089

8. Mironov NM, Aguelon AM, Potapova GI, Omori Y, Gorbunov OV, Klimenkov AA, Yamasaki H: Alterations of (CA)n DNA repeats and tumor suppressor genes in human gastric cancer. Cancer Res 1994, 54:41–44

9. Strickler JG, Zheng J, Shu Q, Burgart LJ, Alberts SR, Shibata D: p53 mutations and microsatellite instability in sporadic gastric cancer: when guardians fail. Cancer Res 1994, 54:4750–4755

10. Wunderlich V, Bottger SM, Graffi A: Preferential alkylation of mitochondrial deoxyribonucleic acid by *N*-methyl-*N*-nitrosourea. Biochem J 1970, 118:99–109

11. Niranjan BG, Bhat NK, Avadhani NG: Preferential attack of mitochondrial DNA by aflatoxin B1 during hepatocarcinogenesis. Science 1982, 215:73–75

12. Blacker JM, Weinstein IB. Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative benzo[a]pyrene. Science 1980, 209:297

13. Allen JA, Coombs MM: Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. Nature 1980, 287:244

14. Clayton DA, Doda JN, Friedberg EC: The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci USA 1974, 71:2777–2781

15. Clayton DA: Replication of animal mitochondrial DNA. Cell 1982, 28:693–705

16. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations and human cancers. Science 1991, 253:49–53

17. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG: Sequence and organization of the human mitochondrial genome. Nature 1981, 290:457–465

18. Shibata D, Hawes D, Li ZH, Hernandez AM, Spruck CH, Nichols PW: Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. Am J Pathol 1992, 141:539–543

19. Cortopassi GA, Arnheim N: Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acids Res 1990, 18:6927–6933

20. Walberg MW, Clayton DA: Sequence and properties of the human KB cell and mouse cell D-loop regions of mitochondrial DNA. Nucleic Acids Res 1981, 9:5411–5421

21. Fisher RP, Topper JN, Clayton DA: Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. Cell 1987, 50:247–258

22. Wallace DC, Lott MT, Torroni A, Shoffner JM: Report of the committee on human mitochondrial DNA. Cytogenet Cell Genet 1991, 58:1103–1123

23. Shoffner JM, Wallace DC: Mitochondrial genetics: principles and practice. Am J Hum Genet 1992, 51:1179–1186

24. Warburg O: On the origin of cancer cells. Science 1956, 123:309–314

25. Morais R, Zinkewich-Peotti K, Parent M, Wang H, Babai F, Zollinger M: Tumor-forming ability in athymic nude mice of human cell lines devoid of mitochondrial DNA. Cancer Res 1994, 54:3889–3896

26. Kunkel TA, Loeb LA: Fidelity of mammalian DNA polymerases. Science 1981, 213:765–767

27. Shay JW, Werbin H: Are mitochondrial DNA mutations involved in the carcinogenic process? Mutation Res 1987, 186:149–160

28. Feig DI, Reid TM, Loeb LA: Reactive oxygen species in tumorigenesis. Cancer Res 1994, 54(suppl):1890s–1894s

29. Monnat RJ, Loeb LA: Nucleotide sequence preservation of human mitochondrial DNA. Proc Natl Acad Sci USA 1985, 82:2895–2899

30. Cortopassi GA, Shibata D, Soong NW, Arnheim N: A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. Proc Natl Acad Sci USA 1992, 89:7370–7374

31. Yamamoto H, Tanaka M, Katayama M, Obayashi T, Nimura Y, Ozawa T: Significance of existence of deleted mitochondrial DNA in cirrhotic liver surrounding hepatic tumor. Biochem Biophys Res Commun 1992, 182:913–920

32. Kovacs G, Welter C, Wilkens L, Blin N, Deriese W: Renal oncocytoma: a phenotypic and genotypic entity of renal parenchymal tumors. Am J Pathol 1989, 134: 967–971

33. Tallini G, Ladanyi M, Rosai J, Jhanwar SC: Analysis of nuclear and mitochondrial DNA alterations in thyroid and renal oncocytic tumors. Cytogenet Cell Genet 1994, 66:253–259

34. Heerdt BG, Chen J, Stewart LR, Augenlicht LH: Polymorphisms, but lack of mutations or instability, in promotor regions of the mitochondrial genome in human colon tumors. Cancer Res 1994, 54:3912–3915

35. Streisinger G, Okada Y, Emrich J, Newton J, Tsugita A, Terzaghi E, Inouye M: Cold Spring Harbor Symp Quant Biol 1966, 31:77–84

36. Mita S, Rizzuto R, Moraes CT, Shanske S, Arnaudo E, Fabrizi GM, Koga Y, DiMauro S, Schon EA: Recombination via flanking direct repeats is a major cause of large-scale deletions of human mitochondrial DNA. Nucleic Acids Res 1990, 18:561–567

37. Ahne A, Muller-Derlich J, Merlos-Lange AM, Kanbay F, Wolf K, Lang BF: Two distinct mechamisms for deletion in mitochondrial DNA of Schizosaccharomyces pombe mutator strains. J Mol Biol 1988, 202:725–734

38. Kolodner RD, Reenan RAG: Characterization of insertion mutations in the Saccharomcyces cerevisiae MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. Genetics 1992, 132:975–985

39. Blot WJ, Devesa SS, Kneller RW, Fraumeni JF: Rising incidence of adenocarcinoma of the esophagus and gastric cardia. JAMA 1991, 265:1287–1289

40. Chang DD, Clayton DA: Priming of human mitochondrial DNA replication occurs at the light-strand promoter. Proc Natl Acad Sci USA 1985, 82:351–355

41. Chang DD, Clayton DA: Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. Cell 1984, 36:635–643

42. Clayton DA: Transcription and replication of animal mitochondrial DNAs. Int Rev Cytol 1992, 141:217–232

43. Novell PC: The clonal evolution of tumor cell populations. Science 1976, 194:23–28

44. Shibata D, Schaeffer J, Li ZH, Capella G, Perucho M: Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not adenocarcinomas. J Natl Cancer Inst 1993, 85:1058–1063

45. Shibata D, Weiss LM: Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol 1992, 140:769–774